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Selective Neurofilament (SMI-32, FNP-7 and N200) Expression in Subpopulations of Layer V Pyramidal Neurons *In Vivo* and *In Vitro*

Courtney C.J. Voelker[1], Nathalie Garin[2], Jeremy S.H. Taylor[1], Beat H. Gähwiler[3], Jean-Pierre Hornung[4] and Zoltán Molnár[1,4]

[1]Department of Human Anatomy and Genetics, University of Oxford, South Park Road, Oxford, OX1 3QX, UK, [2]Swiss Institute for Experimental Cancer Research, Ch. Boveresses 155, 1066 Epalinges, Switzerland, [3]Brain Research Institute, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland and [4]Institut de Biologie Cellulaire et de Morphologie, Rue Du Bugnon 9, 1005 Lausanne, Switzerland

There are two main types of layer V pyramidal neurons in rat cortex. Type I neurons have tufted apical dendrites extending into layer I, produce bursts of action potentials and project to subcortical targets (spinal cord, superior colliculus and pontine nuclei). Type II neurons have apical dendrites, which arborize in layers II–IV, do not produce bursts of action potentials and project to ipsilateral and contralateral cortex. The specific expression of different genes and proteins in these two distinct layer V neurons is unknown. To distinguish between distinct subpopulations, fluorescent microspheres were injected into subcortical targets (labeling type I neurons) or primary somatosensory cortex (labeling type II neurons) of adult rats. After transport, cortical sections were processed for immunohistochemistry using various antibodies. This study demonstrated that antigens recognized by SMI-32, N200 and FNP-7 antibodies were only expressed in subcortical (type I) — but not in contralateral (type II) — projecting neurons. NR1, NR2a/b, PLCβ_1, BDNF, NGF and TrkB antigens were highly expressed in all neuronal subpopulations examined. Organotypic culture experiments demonstrated that the development of neurofilament expression and laminar specificity does not depend on the presence of the subcortical targets. This study suggests specific markers for the subcortical projecting layer V neuron subpopulations.

Keywords: cell differentiation, corpus callosum, intracortical and intercortical connections, spinal cord, superior colliculus

Introduction

The cerebral cortex is comprised of many different neuronal types (Peters and Jones, 1985), which can be classified according to distinct morphology, connectivity, and neurochemical and electrophysiological characteristics; these characteristics can be related to the laminar location of the cell body. Although there is basic structural similarity across the neocortex, specific functions are clearly localized to distinct areas, which are characterized by microcircuitry, input and output connectivity, local cytoarchitecture and proportions of cell types (Brodmann, 1909). The subtle variations in cytoarchitecture reflect important differences in the computational role of numerous cortical areas (Kaas, 1993). Although much research has been conducted on brain development, fundamental questions remain about how specific neuronal subpopulations differentiate and form functional circuits.

Layer V pyramidal neurons provide an excellent model for addressing these important questions. In adult rodent cortex, there are two major subpopulations of layer V pyramidal neurons that have distinct projection targets, somatodendritic morphologies and electrophysiological properties (Larkman and Mason, 1990; Koester and O'Leary, 1992; Kasper *et al.*, 1994). Type I subcortical projecting neurons (projecting to the spinal cord, superior colliculus and pontine nuclei) have tufted apical dendrites terminating in layer I and fire bursts of action potentials in response to depolarizing current. In contrast, type II contralateral cortex projecting neurons have, non-tufted apical dendrites, which arborize in layers II–IV and never fire bursts of action potentials (Kasper *et al.*, 1994). Our main question is, are there molecules that characterize the different somatodendritic morphologies of these two neuronal subpopulations?

The aim of this study was to identify proteins that are differentially expressed in layer V pyramidal neuron subpopulations in the adult rat. Several proteins have been reported to be strongly expressed in layer V, including: (i) neurotransmitter receptors – *N*-methyl-D-aspartate receptor subunit 1 (NR1; Aoki *et al.*, 1994) and NR2a/b (Conti *et al.*, 1999); (ii) neurotrophin receptors – tyrosine kinase receptor type B (TrkB; Tongiorgi *et al.*, 1999; Miller, 2000); (iii) neurotrophins – nerve growth factor (NGF; Miller, 2000) and brain-derived neurotrophic factor (BDNF; Murer *et al.*, 1999); and (iv) neurofilaments – Sternberger monoclonal incorporated antibody 32 (SMI-32; Hof *et al.*, 1995; Gabernet *et al.*, 1999), medium-sized neurofilament clone (FNP-7; Hornung and Riederer, 1999) and neurofilament 200 (N200; Sasaki and Maruyama, 1994). However, these studies did not investigate the protein expression in layer V pyramidal neuron subpopulations based on axonal projection. Therefore, for most of these proteins, it is not clear if all – or only a subset of pyramidal cells – express these molecules. The medium-sized neurofilament proteins (SMI-32, N200 and FNP-7) were of particular interest because previous studies in primate (monkey and human) have shown that these proteins are expressed in ~30% of cortical pyramidal neurons (Campbell *et al.*, 1991; Hof and Morrison, 1995; Hof *et al.*, 1996b; Bussière *et al.*, 2003a). These studies have described the overall regional and laminar distributions of cells containing these neurofilaments, but have only examined corticocortical neurons. This paper further characterizes the neuronal pyramidal populations that express these neurofilament proteins by examining not only the corticocortical – but also the subcortical – projecting neurons in layer V. The only layer V pyramidal cell subpopulation marker identified is the transcription factor Otx1, which is specific for subcortical projecting neurons (Weimann *et al.*, 1999). In this study, layer V pyramidal neurons were divided into subpopulations based on their axonal projection site, which were identified by retrograde labeling. Immunohistochemistry was then used to screen the identified subpopulations using a panel of antibodies

against proteins expressed in layer V. Three proteins were identified that are selectively expressed in subcortical (superior colliculus and spinal cord projecting type I neurons) – but not contralateral cortex (type II neurons) – projecting layer V pyramidal neurons in adult rats. Their specific and selective expression pattern was maintained in early postnatal cortical slice cultures, suggesting that the development and maintenance of these neurofilament protein expression patterns are not dependent on their target.

Materials and Methods

Animals

Nine young adult wistar rats (120–180 g) were used for this study (n = 3, cortical injections; n = 3, spinal cord injections; n = 3, superior colliculus injections). Embryonic day 16 (E16) rats (n = 11) and postnatal day 4 (P4) rats (n = 12) were used for the organotypic cultures. All experimental protocols were approved by and in accordance with the regulations and guidelines of the University of Oxford (UK), the Home Office (UK) and the University of Lausanne (Switzerland).

Retrograde Tracing with Fluorescent Latex Microspheres

Green fluorescent latex microspheres (Lumafluor, Naples, FL) were used to identify subpopulations of cortical layer V pyramidal cells based on their axonal targets.

Adult rats were anesthetized with 2.7 mg/kg Hypnovel (Roche, Basel, Switzerland), Hypnorm (Janssen, Titusville, NJ) and distilled H_2O (1:1:2 volume ratio), which was delivered i.p. and placed in a stereotaxic frame. After the skin was disinfected and incised, a microdrill was used to perform a craniotomy. Glass micropipettes (Clark Electromedical Instruments, Reading, UK) and a binocular stereomicroscope (Zeiss, Germany) were used to inject 0.3–1.0 µl of microspheres into one of three pyramidal cell targets: primary somatosensory cortex (n = 3; 3.0 mm posterior to bregma, 3.5 mm lateral of sagittal suture, 1 mm deep; Welker *et al.*, 1996), superior colliculus (n = 3; 6.5 mm posterior to bregma, 1.5 mm lateral of sagittal suture, 3.5 mm deep; Paxinos *et al.*, 1985) or spinal cord [n = 3; between thoracic 1 (T1) and (T2)]. Each target received two or three injections ~100 µm from each other. The micropipette was kept in place for 1–2 min before retraction. During the postoperative period, animals were kept under a heating lamp before returning them to their cages. All animals recovered quickly and resumed normal behavior following the procedure. Animals were allowed to survive for 24–48 h to permit adequate retrograde transport of the microspheres to the pyramidal soma (Fig. 1*A*). For the organotypic culture experiments, P3 rats were anesthetized by hypothermia and injections into either the somatosensory cortex (n = 6; 2 mm posterior to bregma, 2–3 mm lateral of sagittal suture, approximately 150–200 µm deep) or the center of the superior colliculus (n = 6; under direct visual guidance since it is not yet covered by the occipital pole of the cortex) were made. The rest of the retrograde-labeling procedure for P3 animals followed the adult protocol above.

Immunohistochemistry

After 1–2 days, animals were anesthetized with sodium pentobarbitone (25 mg/kg) delivered i.p. and perfused transcardially with cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and post-fixed for 6 h to overnight in 4% paraformaldehyde in 0.1 M PB (pH 7.4) at 4°C. Brains were sectioned at 60 µm in the coronal plane using a vibroslicer (VT1000S; Leica, Heidelberg, Germany) and serial sections through the primary somatosensory and motor cortices were collected. Free-floating sections were washed in 0.05 M tris buffer containing 0.9% NaCl (TBS; pH 7.4) and incubated for 2 h in TBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 to mask non-specific binding sites. Sections were then incubated overnight in the primary antibody diluted in TBS containing 1% NGS and 0.1% Triton X-100 (see Table 1 for dilutions). After rinsing in TBS, sections incubated with polyclonal primary antibodies were further incubated in cyanine (CY3)-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA) in TBS with 1% NGS for 2 h (see Table 1 for dilutions). Sections incubated with monoclonal primary antibodies (Table 1) were initially incubated in biotinylated-conjugated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) in TBS with 1% NGS (all diluted 1:100) for 2 h, rinsed in TBS and incubated in CY3-conjugated to streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA) for 2 h (all diluted 1:500). After a final rinse in TBS, sections were counterstained with bisbenzimide Hoechst trihydrochloride (2.5 µg/ml PBS; Sigma-Aldrich, St Louis, MO), mounted on gelatin-coated slides, air-dried and cover slipped with PBS. Slides were stored in 4°C and protected from light. The immunohistochemistry controls were negative (data not shown).

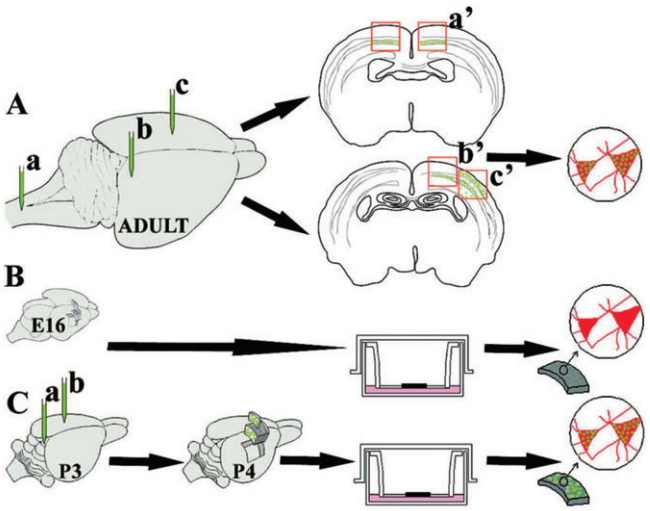

Figure 1. Schematic representation of fluorescent latex microsphere injection and co-culturing methods. (*A*) Fluorescent microsphere injection locations into the (*a*) adult rat spinal cord, (*b*) superior colliculus, or (*c*) contralateral cortex. Microspheres were retrogradely transported from the target tissue location to the cell bodies of layer V pyramidal neurons. Brains were sectioned coronally and prepared for immunohistochemistry with the various antibodies tested. (*a′*) Bilateral distribution of layer V spinal cord projecting neurons. (*b′*) Location of layer V neurons projecting to the ipsilateral superior colliculus. (*c′*) Distribution of the contralateral cortex projecting neurons, which were located in all layers except layer I. Layers II, III and V showed a particularly strong labeling. (*B*) Cortical sections were cultured from E16 rats for 2 weeks. Sections were then fixed in 4% paraformaldehyde and then immunostained with SMI-32 and FNP-7. (*C*) Layer V neurons were pre-labeled before culturing. Microsphere injections were made into the (*a*) superior colliculus or (*b*) contralateral cortex of P3 animals. Cortical slices containing back-labeled layer V neurons were cultured at P4 for 2 weeks, fixed and immunostained with SMI-32 and FNP-7 as in *B*.

Organotypic Cortical Cultures

To determine if targets of layer V pyramidal neurons are responsible for the maintenance and the selective expression of neurofilament antigens, two types of organotypic culture experiments were performed (Fig. 1*B*,*C*).

Cortical slices were prepared from the parietal cortex of E16 rats (n = 11) and maintained for 2 weeks in culture conditions according to the methods described in Molnár and Blakemore (1991, 1999; see also Fig. 1*B*). A tissue chopper was used to cut 350 µm thick cortical slices from E16 rats. The tissue blocks were transferred to a Petri dish containing Hanks balanced salt solution (Sigma) supplemented with glucose to a final concentration of 6.5 mg/ml. Explants were then placed on Transwell-COL culture chamber membranes (pore size 0.4 µm, 24.5 mm diameter inserts; Costar, Cambridge, MA). Cultured explants were maintained in N2 medium [1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12, supplemented with insulin (5 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (100 mM) and selenium (30 nM, as Na_2SeO_4)]. These constituents were made up into stock solutions, filter-sterilized (pore size 0.2 µm) and then added to the medium individually. In most cases, we used N2 stock solution (Gibco BRL, Grand Island, NY). The

Table 1
Sources, technical details and concentrations of antibodies used

Antigen	Source	Type	Specificity	Dilutions			
				PAb	bGM	Cy3-St	Cy3-R
NR1	C.I.I.	R IgG pAb	R1-1a, -1b, -2a, -2b subunits of the NMDA receptor	1:500			1:100
NR2a/b	C.I.I.	R IgG pAb	R2a and R2b subunits of the NMDA receptor	1:1000			1:500
PLCb1	S.C.B.I.	R IgG pAb	PLCb1 enzyme of the PLC isoenzyme family	1:1000			1:100
BDNF	S.C.B.I.	R IgG pAb	Amino terminus of BDNF	1:500			1:100
NGF	S.C.B.I.	R IgG pAb	Amino terminus of NGF	1:1000			1:500
TrkB	S.C.B.I.	R IgG pAb	TrkB receptor of the Trk family	1:200			1:500
SMI-32	S.M.I.	M IgG mAb	Non-phos. NF-H (200 kDa)	1:5000	1:100	1:500	
N200	S.A.	M IgG mAb	Phos. and non-phos. NF-H (200 kDa)	1:400	1:100	1:500	
FNP-7	V.L.	M IgG mAb	Non-phos. NF-M (150 kDa)	1:1200	1:100	1:500	

Abbreviations: BDNF, brain derived neurotrophic factor; bGM, biotinylated goat anti-mouse antibody; C.I.I. Chemicon International Inc.; Cy3-R, Cy3 goat anti-rabbit antibody; Cy3-St, Cy3 goat anti-streptavidin antibody; FNP-7, medium-sized neurofilament clone; M, mouse; mAb, monoclonal antibody; N200, neurofilament 200 kDa; NF, neurofilament; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; NR1, *N*-methyl-D-aspartate receptor subunit 1; NR2a/b, *N*-methyl-D-aspartate receptor subunit 2a/b; pAb, polyclonal antibody; PAb, primary antibody; PLCb1, phospholipase C isoenzyme beta1; R, rabbit; S.A. Sigma-Aldrich Corp. (St Louis, MO); S.C.B.I. Santa Cruz Biotechnology Inc. (Santa Cruz, CA); S.M.I. Sternberger Monoclonal Inc. (Lutherville, MD); SMI-32, Sternberger Monoclonal Inc. antibody 32; TrkB, tyrosine protein kinase receptor type B (Temecula, CA); V.L. Virginia Lee (Philadelphia, PA).

cultures were maintained in Transwell-COL culture chambers (Costar) under standard culturing conditions with continuous flow of humidified carbogen (5% CO_2 and 95% air, 100% humidity, 40°C) in a modular incubator chamber (Flow Laboratories). After culturing, the slices were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.3) and the entire slice (without resectioning) was immunostained using SMI-32 and FNP-7 antibodies (for details, see above).

Organotypic culture experiments with cortical slices containing pre-labeled pyramidal cells were used to examine whether the subpopulations of layer V neurons maintain their antigenicity in the absence of the target tissue (Fig. 1*C*). At P3, after the corticofugal projections have reached the superior colliculus and the contralateral cortex, fluorescent latex microspheres were injected under direct visual guidance into either the somatosensory cortex or the superior colliculus in rats as described above. At P4, animals were anesthetized by hypothermia and cortical slice cultures (40 slices in total) were prepared (Porter *et al.*, 1999) from regions containing labeled cells (Fig. 1*C*). The brains were quickly removed from the skull and placed into cold, sterile MEM (Life Technologies, Gaithersburg, MD) with 200 mM Tris buffer (dissecting medium). A McIlwain tissue chopper was used to cut sections at a thickness of 250 μm and placed into dissecting medium. The slices were then placed into a Millicell-CM (Millipore, Bedford, MA) insert that was immersed in dissecting medium. Dissecting medium was replaced with culture medium, which included: DMEM supplement with 25% normal horse serum (NHS; Life Technologies) and 20 nM glutamine. Medium levels were maintained so that there was an air-medium interface. Medium was refreshed every 2–3 days by removing half the volume and replacing it with fresh medium. Slices were cultured for 2 weeks after which they were fixed and immunostained without resectioning using FNP-7 and SMI-32 (see above). Individual microsphere-labeled layer V neurons were examined for immunohistochemistry using fluorescence and confocal laser scanning microscopy.

Quantification of Double-labeled Cells using Fluorescence Microscopy

Immunolabeled sections from adult, embryo and neonate animals were analyzed with a Leitz Diaplan fluorescence microscope (Wetzlar, Germany) equipped with appropriate barrier filters for the various fluorophores. Quantification of double-labeled cells was restricted to the somatosensory cortex for superior colliculus and contralateral cortex projecting cells and to the motor cortex for the spinal cord projecting neurons. Two to three cortical sections for each injection paradigm were selected that contained numerous fluorescent back-labeled pyramidal cells within S1 and M1. All layer V pyramidal neurons per section that contained fluorescent microspheres were examined for immunostaining by changing the fluorescence filter under 100× oil immersion objective. True color images were captured using a Leica DC 500 digital camera (Leica, Bensheim, Germany). The present study is based on the examination of 6501 layer V pyramidal neurons. An average of 240 cells per layer V subpopulation was counted for each antibody (n = 9) used. All layer V neurons per postnatal organotypic culture that contained fluorescent microspheres were examined for neurofilament immunoreactivity. In total, 40 cultures were prepared from somatosensory cortex injections (n = 6 rats; SMI-32 = 320 cells counted; FNP-7 = 286 cells counted) and superior colliculus injections (n = 6 rats; SMI-32 = 196 counted; superior colliculus = 361 cells counted).

Statistical Analysis

A total of nine adult rats were used for the immunohistochemical and back-labeling protocol (Fig. 1*A*); three for each injection paradigm (n = 3, somatosensory cortex; n = 3, superior colliculus; n = 3, spinal cord). A total of 12 P3 rats were used for the culture experiments (Fig. 1*B*,*C*); six for each injection paradigm (n = 6, somatosensory cortex; n = 6, superior colliculus). The percentage of double-labeled cells identified relative to the total number of cells counted (fluorescent-labeled only plus double-labeled) was calculated for each animal. Percentages for each subpopulation (contralateral cortex, superior colliculus or spinal cord projecting cells) were compared for each antibody using a student's t-test, with $P < 0.05$ being considered statistically significant.

Confocal Microscopy

Laser-scanning confocal microscopy (Leica TCS NT, Germany) validated the quantification conducted under the fluorescence microscope. Excitation was obtained with an argon–krypton laser, with lines set at 488 nm for fluorescein isothiocyanate (FITC) and 568 nm for tetramethyl rhodamine isothiocyanate (TRITC). Between six and eight optical sections (z distance between each: 0.2–0.5 μm) were scanned through a single pyramidal cell. Images were taken in a 1024 × 1024 pixel format using a 100×/1.4 N.A. oil immersion objective. Individual optical sections and the Z-axis reconstructions were examined before images were compiled into a single image. The single images were then processed using Adobe Photoshop 6.0.

Results

Distribution of Labeled Cells from the Fluorescent Microsphere Injections into Different Pyramidal Cell Targets

Spinal Cord Projecting Neurons

Spinal cord injections (Fig. 2*A*) revealed retrogradely labeled cells in the somatosensory and motor cortices (Fig. 2*B*,*C*). Cells were more numerous in the dorso-medial part of the frontal poles. The labeled neurons were limited to layer V (Fig. 2*B*,*D*). The soma size of the spinal cord projecting neurons appeared similar to that of the superior colliculus projecting neurons, both of which were larger than those projecting to the contralateral hemisphere (Fig. 2*C*,*G*,*K*).

Superior Colliculus Projecting Neurons

All cells labeled after superior colliculus injections (Fig. 2*E*) were located exclusively in layer V (Fig. 2*F*,*H*). The majority of back-labeled cells were found in the upper part of layer V. A minority of labeled cells were scattered in the lower portion of layer V as well; however, no sharp boundary between the upper and lower layer V cells was observed. The band of labeled cells extended throughout the primary somatosensory cortex, while more posterior regions (i.e. occipital cortex) contained a slightly greater number of labeled neurons.

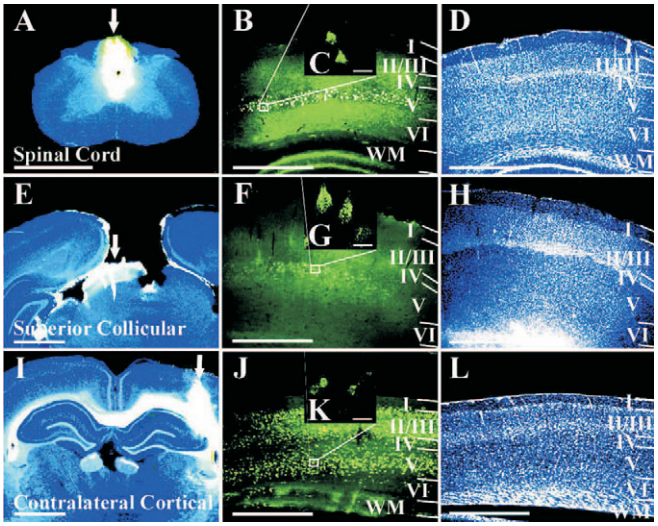

Figure 2. Labeling of layer V pyramidal cells by fluorescent latex microsphere injections into the spinal cord, superior colliculus and contralateral cortex hemisphere. Injection sites into the (*A*) spinal cord, (*E*) superior colliculus and (*I*) primary somatosensory cortex on coronal sections (indicated by arrows; white-green appearance) counterstained with bisbenzimide (blue appearance). Coronal cortical sections show microsphere labeling of pyramidal neurons after retrograde transport (*B*, *C*, *F*, *G*, *J*, *K*). Sections were counterstained with bisbenzimide to reveal the cytoarchitecture for cortical layer identification (*D*, *H*, *L*). (*B*) Neurons projecting to the spinal cord were labeled and (*D*) were restricted to layer V. (*C*) A confocal image of fluorescent microbeads contained in layer V pyramidal neurons taken from *B* after a spinal cord injection. (*F*) superior colliculus injections label pyramidal neurons in the ipsilateral cortex and (*H*) were also exclusively found in layer V. (*G*) A confocal image of fluorescent microbead labeled neurons taken from *F* after a superior colliculus injection. (*J*) Pyramidal neurons projecting to the contralateral cortex hemisphere through the corpus callosum and (*L*) were located in all cortical layers except layer I. Layers II, III and V showed the greatest number of cells projecting to the contralateral cortex. (*K*) A confocal image of layer V neurons labeled with fluorescent microbeads taken from *J* after a contralateral cortex injection. (*D*, *H*, *L*) Images were taken from the same field shown in *B*, *F* and *J* with a UV filter. Scale bars = 2 mm (*A*, *E*, *I*); 1 mm (*B*, *D*, *F*, *H*, *J*, *L*); 20 μm (*C*, *G*, *K*).

Contralateral Cortex Projecting Neurons in the Primary Somatosensory Area

Although it is known that primary cortical areas have fewer callosal connections (Akers and Killackey, 1978), numerous contralateral projecting neurons were found in the primary somatosensory cortex after cortical injections (Fig. 2*I*). The labeled cells were distributed in all cortex layers, with the exception of layer I (Fig. 2*J*,*L*). Layers II, III and V had the greatest number of labeled cells, whereas layer IV contained relatively few labeled cells. The callosal projecting neurons were smaller than the spinal cord or superior colliculus projecting neurons (Fig. 2*K*).

Immunohistochemical Analysis of Layer V Projection Neuron Subpopulations

Markers Ubiquitously Expressed in all Three Subpopulations Examined

Most neurons in the neocortex were stained with the antibody against the glutamate receptor, NR-1. However, layers II, III, V and VI contained especially large numbers of heavily labeled cells bodies. The apical dendrites, as well as some parts of the terminal tufts, exhibited strong immunoreactivity. NR2a/b was also expressed in all cortical layers, with cell bodies most strongly labeled in layers II–V. Similar to NR-1 staining, NR2a/b also stained the proximal dendrites. The isozyme, phospholipase $C\beta_1$ ($PLC\beta_1$) was diffusely expressed in layers II, III, IV, V and VI on neuronal apical dendrites. Staining for the neurotrophin BDNF showed expression in cortical layers II, III, V and VI throughout the cortex with labeling of the soma, proximal dendrites and axons of pyramidal cells. NGF was expressed in almost every cell in the cortex with a particularly high density in layers II, III and V. Pyramidal cell bodies and dendrites were most strongly immunolabeled. The BDNF receptor, TrkB was expressed in large numbers of neurons in all cortical layers. Layers V and VI contained the greatest number of immunoreactive cells. Only the initial segment of the layer V neuronal apical dendrites and primary dendrites were labeled. The apical dendrites and the apical tufts were not immunoreactive for TrkB.

Markers Specific for Subcortical Projecting Pyramidal Neurons (SMI-32, FNP-7 and N200)

SMI-32, which reacts with non-phosphorylated epitopes in neurofilament-M (150 kDa) and -H (200 kDa; Lee *et al.*, 1988), was intensely expressed in the cortical white matter. Layers II, III and V cells were strongly stained in the medial somatosensory cortex; however, the immunoreactivity in layers II and III decreased when progressing laterally, while layer V staining remained the same (Fig. 3*A*,*B*). SMI-32 was expressed in the soma and the dendrites, but only some thick axons of pyramidal neurons (Fig. 3*C*,*D*). FNP-7, which reacts exclusively with non-phosphorylated NF-M, is most prominently expressed in layers III, V and VI (Fig. 3*E*,*F*). FNP-7 also stained the somata and apical and basal dendrites, as well as the proximal axon extending into the white matter (Fig. 3*G*,*H*). N200, which reacts with both the phosphorylated and non-phosphorylated NF-H, most strongly stained layers III, V and VI (Fig. 3*I*,*J*). Like SMI-32 and FNP-7, N200 stained apical and basal

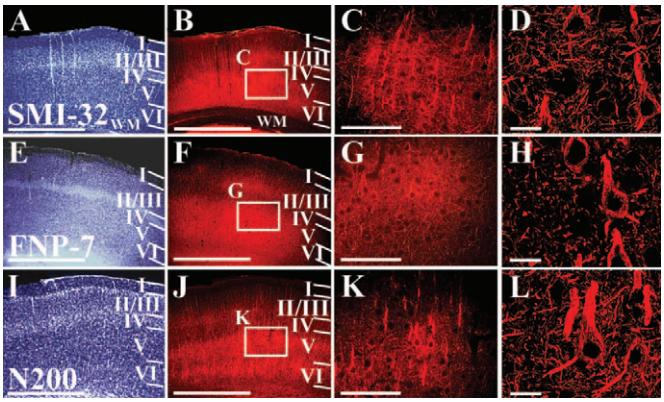

Figure 3. Laminar distribution of neurofilament protein immunoreactivity in the primary somatosensory cortex. (*A–D*) SMI-32, (*E–H*) FNP-7 and (*I–L*) N200 all showed an intense staining pattern in layers II–III, V, and VI. (*C*, *D*) Higher magnifications of layer V pyramidal cells showed SMI-32 immunoreactivity on the soma and apical and basal dendrites. (*G*, *H*) High power confocal images of layer V neurons immunoreactive for FNP-7. (*K*, *L*) Higher magnification of N200 immunoreactive layer V neurons. Scale bars = 1 mm (*A*, *B*, *E*, *F*, *I*, *J*); 100 μm (*C*, *G*, *K*); 20 μm (*D*, *H*, *L*).

dendrites as well as a few thick axons (Fig. 3*K*,*L*). Immunostaining from all three neurofilament markers indicated that the majority of stained fibers that exited the cortex entered the cortical white matter and turned laterally toward the internal capsule (not toward the corpus callosum). Nevertheless, strongly immunoreactive fibers were observed within the corpus callosum itself and layers II–III consistently showed a very small percentage of contralateral cortex projecting cells which contained both green microspheres and stained with one of the three neurofilament antibodies (average ± SEM; SMI-32 = 2.31 ± 0.58; N200 = 8.89 ± 2.27; FNP-7 = 1.74 ± 0.39). Other fiber tracts — including the cerebral peduncle and the optic tract — as well as the thalamic reticular nucleus were also heavily stained. A sizable percentage of corticocortical neurons projecting to ipsilateral cortical areas showed neurofilament reactivity (SMI-32 = 21.8% and FNP-7 = 61%). This finding suggests that a large proportion of the cells that establish intracortical connections also express SMI-32 and FNP-7; therefore, these markers are not restricted to the longer-range intercortical and subcortical projection neurons.

Quantification of Immunoreactive Layer V Pyramidal Cells Based on their Projection Sites

Markers Ubiquitously Expressed in all Three Subpopulations Examined

Layer V pyramidal cells containing fluorescent microspheres were quantified based on their immunoreactivity. Figure 4 shows confocal images of the three cell populations (contralateral cortex, superior colliculus and spinal cord projecting) stained with NR1, NR2a/b, PLCβ_1, BDNF, NGF and TrkB antibodies. The panels illustrate that all six antibodies co-localized with the pyramidal cells that contained fluorescent microspheres labeled from the spinal cord, superior colliculus and contralateral cortex. The percentage of double-labeled cells compared to the total number of cells counted (fluorescent-labeled only plus double-labeled) was calculated. Regardless of their projection site, a very high percentage of back-labeled

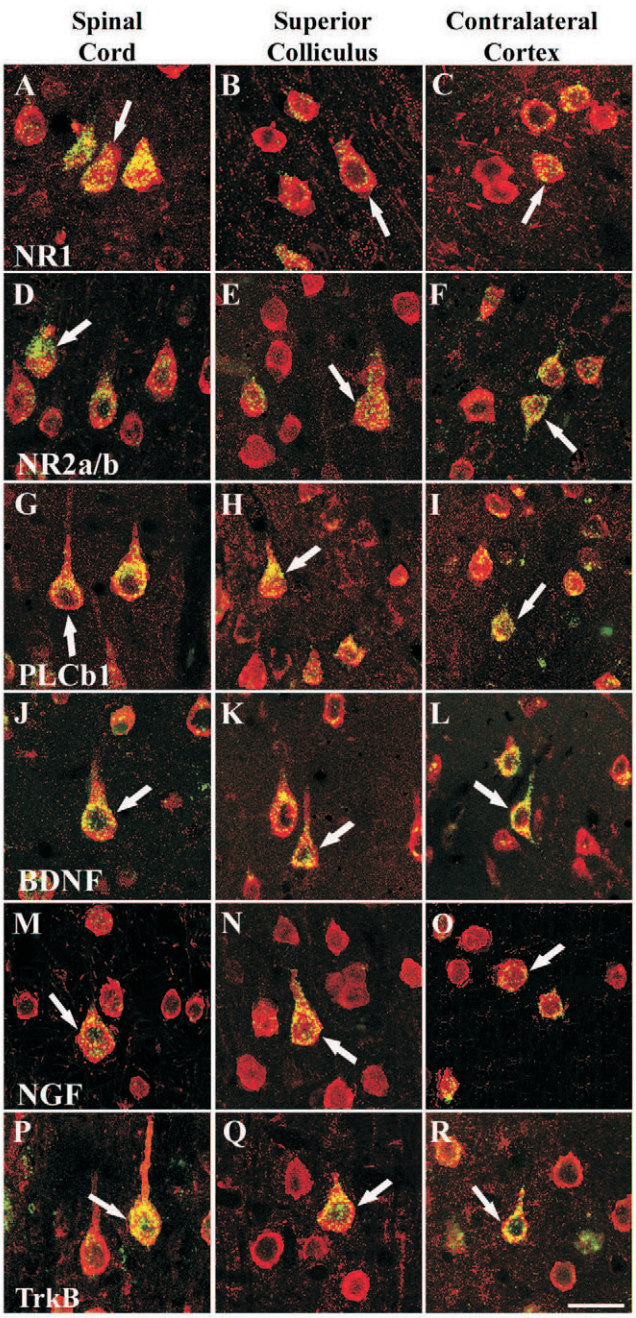

Figure 4. Detection of immunoreactivity in layer V pyramidal cell populations back-labeled from three different targets. Confocal microscopic images of layer V pyramidal neurons showing labeling with latex fluorescent microspheres (green) and immunostaining (red) for various antigens after labeling from the spinal cord (*A*, *D*, *G*, *J*, *M*, *P*), superior colliculus (*B*, *E*, *H*, *K*, *N*, *Q*) and contralateral cortex (*C*, *F*, *I*, *L*, *O*, *R*). (*A–C*) NR1, (*D–F*) NR2a/b, (*G–I*) PLCβ_1, (*J–L*) BDNF, (*M–O*) NGF and (*P–R*) TrkB antibodies. All six of these antibodies produced staining on layer V neurons containing retrogradely transported microspheres (arrows). In the adult rat neocortex, these selected proteins were expressed in all three subpopulations of layer V pyramidal cells: that is, neurons with projections to the spinal cord, superior colliculus and contralateral cortex. Scale bar = 20 μm.

layer V pyramidal cells expressed NR-1, NR2a/b, PLCβ_1, BDNF, NGF and TrkB (Table 2). There was no significant difference between the expression of these six proteins in the three pyramidal cell populations.

Table 2
Proportion of the double-labeled layer V projection neurons back-labeled from different targets and stained with various antibodies

	Spinal cord			Superior colliculus			Contralateral cortex		
Antigen	DLN	Total	Av. %DLN ± SEM	DLN	Total	Av. %DLN ± SEM	DLN	Total	Av. %DLN ± SEM
NR1	218	222	98.67 ± 1.33	208	212	98.16 ± 1.11	223	224	99.37 ± 0.66
NR2a/b	197	198	99.73 ± 0.27	261	266	97.87 ± 0.70	273	275	99.28 ± 0.36
PLCb1	165	168	98.65 ± 1.53	248	255	97.37 ± 1.78	223	227	98.21 ± 0.96
BDNF	207	207	100.00 ± 0.00	354	357	99.31 ± 0.38	243	244	99.63 ±0.37
NGF	179	180	99.57 ± 0.42	372	374	99.64 ± 0.36	229	230	99.48 ± 0.52
TrkB	165	165	100.00 ± 0.00	248	250	98.86 ± 0.60	265	269	98.48 ± 0.96
SMI-32	154	159	97.35 ± 1.33	285	290	98.27 ± 0.01	**2**	**219**	**1.06 ± 0.54**
N200	270	275	98.08 ± 1.04	190	195	98.24 ± 1.00	**5**	**312**	**2.10 ± 1.36**
FNP-7	205	209	98.27 ± 0.46	244	246	97.99 ± 1.01	**3**	**291**	**0.33 ± 0.33**

$n = 3$ adult rats for each injection paradigm (spinal cord, superior colliculus and contralateral cortex). All layer V neurons containing green fluorescent microspheres in each slice were counted ('Total' column). The numbers of layer V cells that contained green fluorescent microspheres and double-labeled with a given antibody (DLN column) were quantified. The percentage of DLN for each animal was calculated. The percentages were averaged and the standard error of the mean was calculated ('Av. %DLN ± SEM' column). Note that ~100% of the spinal cord, superior colliculus and contralateral cortex projecting neurons expressed NR1, NR2a/b, PLCb1, BDNF, NGF and TrkB. The difference between the three populations of cells was not statistically significant for these antibodies. However, the immunoreactivity for the neurofilament markers (SMI-32, N200 and FNP-7) was selectively expressed in subcortical projecting cells (i. e. projecting to the spinal cord and superior colliculus). Virtually none of the contralateral cortex projecting neurons expressed these three neurofilament markers. The difference between the subcortical and callosal projecting cells was statistically significant. SMI-32 SpC versus CC ($P = 0.00038$); SMI-32 SC versus CC ($P = 0.00014$); N200 SpC versus CC ($P = 0.00025$); N200 SC versus CC ($P = 0.00042$); FNP-7 SpC versus CC ($P = 0.0000024$); FNP-7 SC versus CC ($P = 0.00016$). Abbreviations: Av. %DL, average percentage of double-labeled layer V neurons quantified; CC, contralateral cortex projecting neurons; DLN, double-labeled layer V neuron; Total, total number of layer V neurons quantified; SC, superior colliculus projecting neurons; SEM, standard error of the mean; SpC, spinal cord projecting neurons.

Markers Specific for Subcortically Projecting Pyramidal Neurons (SMI-32, FNP-7 and N200)

Figure 5 shows confocal images of the three cell populations (contralateral cortex, superior colliculus and spinal cord projecting) stained with three antibodies (SMI-32, N200 and FNP-7) that are specific for different neurofilament epitopes. The panels demonstrate that SMI-32, N200 and FNP-7 were expressed in neurons projecting to the spinal cord and to the superior colliculus, but these three antigens were not expressed in the neurons projecting through the corpus callosum to the contralateral hemisphere. Sections stained with these three antibodies were analyzed using the same quantification technique described above. Most of the subcortical projecting pyramidal cells expressed SMI-32 (mean ± SEM; spinal cord = 97.35 ± 1.33%; superior colliculus = 98.27 ± 0.007%), N200 (spinal cord = 98.08 ± 1.04%; superior colliculus = 98.24 ± 1.00%) and FNP-7 (spinal cord = 98.27 ± 0.46%; superior colliculus = 97.99 ± 1.01%; Table 2). There was no significant difference between the percentage of cells expressing these three neurofilament markers in the subcortical projecting neurons. However, the contralateral cortex projecting cells barely expressed a quantifiable level of these neurofilament markers (SMI-32 = 1.06 ± 0.54%; N200 = 2.10 ± 1.36%; FNP-7 = 0.33 ± 0.33%; Table 2). The percentage difference between neurofilament expression in callosal – compared to subcortical – projecting neurons was statistically significant (Table 2).

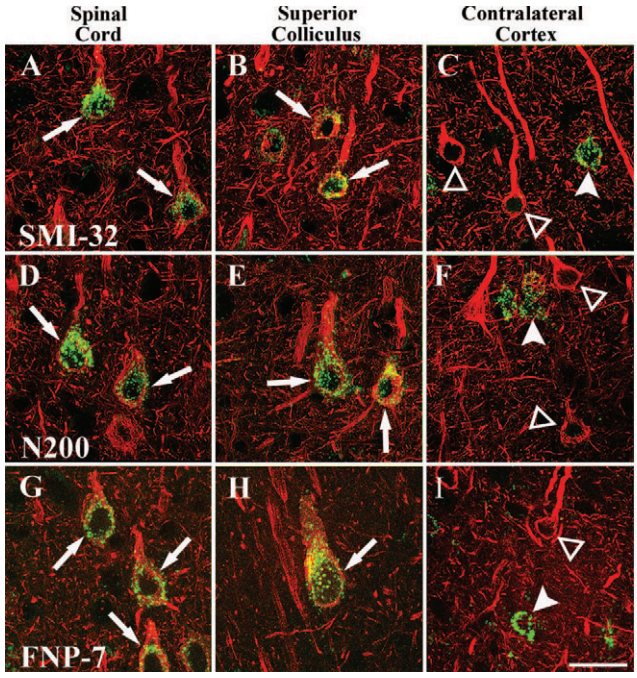

Figure 5. Confocal microscopic images of layer V pyramidal neurons showing that the three neurofilament antibodies (red) specifically stained neurons that project subcortically, but they did not stain cells with projections to the contralateral cortex (all populations contain green microspheres). SMI-32 (*A*, *B*), N200 (*D*, *E*) and FNP-7 (*G*, *H*) were specifically located in the spinal cord (*A*, *D*, *G*) and superior colliculus (*B*, *E*, *H*) projecting layer V neurons (arrows indicate examples of double-labeling). (*C*, *F*, *I*) contralateral cortex projecting neurons (green microspheres indicated by arrowheads), did not express SMI-32 (*C*), N200 (*F*) or FNP-7 (*I*). The open arrow heads indicate neurons expressing the neurofilaments, but do not contain green microspheres and thus do not project to the contralateral cortex. Scale bar = 20 μm.

Neurofilament Protein Expression in Organotypic Culture

We performed two experiments to determine the likelihood that the neurofilament immunoreactivity in layer V projection neurons is elicited and maintained by their subcortical targets through their specific axonal projections. We prepared cortical slices from the parietal cortex of E16 embryos and maintained them in organotypic cultures for two weeks (Fig. 1*B*) to test if layer V neurons express SMI-32 and FNP-7 while developing

without contact with their target tissues. The slices derived from the embryonic cortex did, in fact, express SMI-32 (Fig. 6*A*) and FNP-7 (Fig. 6*B*). In high power images, layer V pyramidal neurons were clearly identified by strong SMI-32 (Fig. 6*C*) and FNP-7 (Fig. 6*D*) immunoreactivity. Layers II and III did not develop as well as the other layers, which is a typical observation in embryonic explants grown in culture (Molnár and Blakemore, 1999).

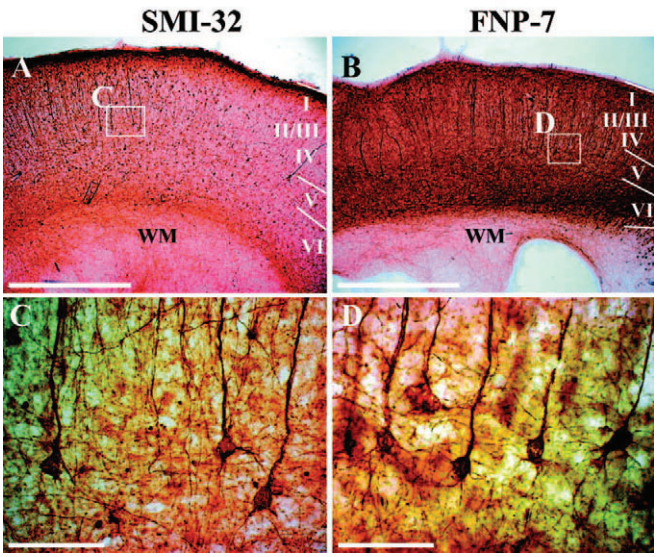

Figure 6. Organotypic cultures of E16 rat cortices express SMI-32 and FNP-7 immunoreactivity in their layer V pyramidal neurons after 2 weeks *in vitro*. The presence of SMI-32 and FNP-7 immunoreactive layer V neurons suggests that the expression of these neurofilaments does not depend on the presence of the subcortical target tissues. (*A*) SMI-32 was expressed in layer V pyramidal neurons *in vitro*. (*C*) Higher magnification of neurons taken from *A*. (*B*) FNP-7 was expressed in layer V pyramidal neurons *in vitro*. (*D*) Higher magnification of neurons taken from *B*. Scale bars = 1 mm (*A*, *B*); 100 μm (*C*, *D*).

To further investigate whether or not subpopulations of layer V neurons maintain their SMI-32 and FNP-7 immunoreactivity specificity in the absence of contact with their target tissue, pyramidal cells were pre-labeled and cortical slices were cultured. At P3, after the corticofugal projections had reached the superior colliculus and the contralateral cortex, fluorescent latex microspheres were injected into one of these targets. At P4, cortical slice cultures were prepared from regions containing labeled cells. These slices were cultured in isolation for two weeks, then fixed and immunostained for FNP-7 and SMI-32 without resectioning (Fig. 7). In 40 cultures, individual microsphere-labeled layer V neurons (n = 1163) were examined for immunohistochemistry. Of the 320 layer V cells that were labeled from the contralateral hemisphere (Fig. 7), only a small percentage (mean ± SEM; 1.69 ± 1.40) expressed SMI-32. In contrast, of the 196 layer V cells that were labeled from the superior colliculus (Fig. 7), a much higher percentage (27.11 ± 4.67) of labeled cells were SMI-32 immunoreactive. Of the 286 layer V neurons that were labeled from the contralateral cortex (Fig. 7), only 4.2% were FNP-7 immunoreactive, whereas 44.6% of layer V neurons (n = 361) that were labeled from the superior colliculus were immunoreactive (Fig. 7). Laminar specificity of neurofilament immunoreactivity was also maintained with stronger staining in layers II, III, V and VI (Fig. 7). Although the absolute number of double-labeled neurons in the cultures was decreased, the overall pattern of neurofilament expression was consistent with the *in vivo* observations.

Discussion

Differential Protein Expression in Layer V Pyramidal Cell Subpopulations

In this study, a combination of retrograde labeling and immunostaining techniques were used to determine whether or not subpopulations of layer V pyramidal cells projecting to distinct

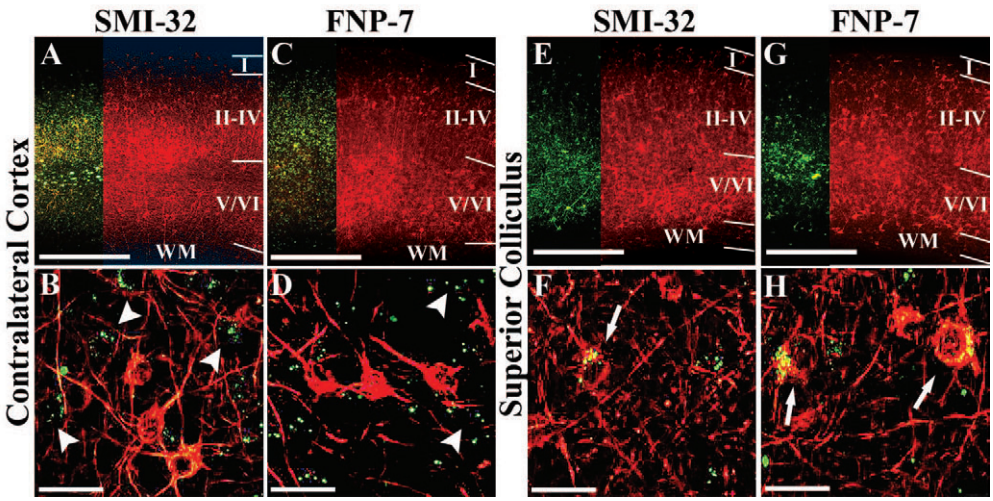

Figure 7. The subpopulation-specific expression of SMI-32 and FNP-7 immunoreactivity is maintained in organotypic cortical cultures from postnatal rats after 2 weeks *in vitro*. Layer V pyramidal neurons were back-labeled from the contralateral cortex (see inserts in *A*, *C*) or from the superior colliculus (see inserts in *E*, *G*) at P3 (both populations contain green microspheres). At P4, the somatosensory cortex of both groups was cultured for 2 weeks. SMI-32 and FNP-7 maintained a laminar (*A*, *C*, *E*, *G*) pattern of expression similar to that found *in vivo*. This suggests that the presence of specific targets is not necessary for the maintenance of the particular layer V populations after P4. (*B*, *D*) Confocal images of pyramidal cells taken from *A* and *C* respectively. Both SMI-32 (*B*) and FNP-7 (*D*) failed to co-localize with contralateral cortex projecting neurons (green microspheres indicated by the arrowheads). (*F*, *H*) Confocal images of pyramidal cells taken from *E* and *G* respectively. A large percentage of both SMI-32 (*F*) and FNP-7 (*G*) co-localized with superior colliculus projecting neurons (indicated by the arrows). P, postnatal day. Scale bars = 1 mm (*A*, *C*, *E*, *G*); 20 μm (*B*, *D*, *F*, *H*).

targets show differential expression of antigens known to be located in layer V of adult rats. These antigens include the following: NR1, NR2a/b, PLCβ_1, BDNF, NGF, TrkB, SMI-32, FNP-7 and N200. Staining patterns found in this study confirm previous observations with the same antibodies: NR1 (Aoki *et al.*, 1994), NR2a/b (Conti *et al.*, 1999), TrkB (Tongiorgi *et al.*, 1999; Miller, 2000), NGF (Miller, 2000), BDNF (Murer *et al.*, 1999), SMI-32 (Hof and Morrison, 1995; Gabernet *et al.*, 1999), FNP-7 (Hornung and Riederer, 1999) and N200 (Sasaki and Maruyama, 1994). However, these experiments show for the first time a striking difference in neurofilament expression (SMI-32, FNP-7 and N200) between subcortical (superior colliculus and spinal cord projecting type I neurons) and contralateral cortex (type II neurons) projecting neurons. While a large proportion of spinal cord and superior colliculus projecting neurons were heavily immunoreactive for the neurofilament antigens (SMI-32, FNP-7 and N200), a visual assessment indicated that none of the callosal projecting layer V cells had significantly detectable immunoreactivity. In contrast, all of the other proteins tested (NR1, NR2a/b, PLCβ_1, BDNF, NGF and TrkB) were expressed equally in the layer V pyramidal subpopulations examined.

Differential Neurofilament Expression Layer V Pyramidal Neuron Subpopulations

Neurofilaments (NF), which are part of the intermediate filament family, are one of the earliest recognizable features of the developing central nervous system (Ulfig *et al.*, 1998). Neurofilaments are heteropolymers consisting of three subunit proteins: NF-L (68 kDa), NF-M (150 kDa) and NF-H (200 kDa) (Hoffman and Lasek, 1975; Nixon and Sihag, 1991; Nixon, 1998). These subunits combine to form filaments (~10 nm diameter). All triplets consist of two structural domains: an α-helical domain, which is the functional backbone and a carboxy end. The carboxy terminals vary in their amount of phosphorylation, which is responsible for the different molecular weights (Shaw, 1991). Decreases in neurofilament expression or abnormal neurofilament phosphorylation have been implicated in normal aging (Vickers *et al.*, 1992; Budinger *et al.*, 2000) as well as in neurological diseases (Bickford *et al.*, 1998) such as: Alzheimer's disease (Morrison *et al.*, 1987; Hof *et al.*, 1990; Hof and Morrison, 1990; Trojanowski *et al.*, 1993; Vickers *et al.*, 1994; Bussière *et al.*, 2003a,b), Parkinson's disease (Goldman *et al.*, 1983), Pick's disease (Perry *et al.*, 1987), amyotrophic lateral sclerosis (Manetto *et al.*, 1988; Munoz *et al.*, 1988; Mizusawa *et al.*, 1989; Tsang *et al.*, 2000), traumatic brain injury (Saatman *et al.*, 1998) and multiple sclerosis (Trapp *et al.*, 1998).

Neurofilaments have a very specific laminar distribution pattern in the cortex that reflects the functional and anatomical brain divisions. Therefore, neurofilament staining is a powerful tool for confirming and extending previous architectural observations (Hof *et al.*, 1995; Preuss *et al.*, 1997; Geyer *et al.*, 2000). SMI-32 recognizes the non-phosphorylated NF-H form, N200 recognizes the non-phosphorylated and phosphorylated NF-H forms and FNP-7 recognizes the non-phosphorylated NF-M form. These protein epitopes initially appear at P7, which interestingly coincides with the divergence of the layer V pyramidal neuronal subpopulations with respect to their somatodendritic morphologies (Riederer and Matus, 1985; Riederer, 1995; Riederer *et al.*, 1995; Kogan *et al.*, 2000).

It is important to relate the onset of neurofilament expression to other aspects of layer V pyramidal cell development. Neurons from layer V subpopulations are born around E15–18 and migrate to layer V by E19–20 (Miller, 1988). Morphologically, they initially appear indistinguishable: all have stout apical dendrites with terminal tufts in layer I and they do not fire action potential bursts in response to depolarizing current (Kasper *et al.*, 1994). Shortly after reaching layer V, they begin extending their axons toward different targets. The callosal axons start to cross the corpus callosum at E16–18, whilst the corticofugal axons enter the internal capsule at E17 and enter the cerebral peduncle at E19. The axons reach the basal pons at E19 and the superior colliculus and spinal cord shortly after birth (De Carlos and O'Leary, 1992). Axonal target invasion occurs postnatally. It is only after axonal outgrowth that the dendritic morphologies begin to diverge (P5; Koester and O'Leary, 1992). The different electrophysiological properties are first detected much later (P14; Kasper *et al.*, 1994). These findings raise the question: how do seemingly similar cell populations differentiate into a heterogeneous group with different targets?

It is not clear what causes these morphological changes, but it has been shown that neurofilaments participate in the structural organization and stabilization of dendrites (Riederer and Matus, 1985; Riederer *et al.*, 1995; Kogan *et al.*, 2000). Therefore, the neurofilament quantity may be important in establishing and maintaining different morphologies of layer V neuronal subpopulations; therefore, playing a role in connections reaching their developmental endpoint (Liu *et al.*, 1994; Kogan *et al.*, 2000).

Previous studies in macaque monkeys have shown that SMI-32 is differentially expressed in certain subpopulations of pyramidal cells. These studies have exclusively examined corticocortical – not subcortical – projecting neurons in layers II, III, V and VI. The corticocortical neuron populations that express low levels of SMI-32 include cells that project from the anterior cingulate to prefrontal cortex; from prefrontal to prefrontal cortex; and from V1, V2 or V3 to V4 (Campbell *et al.*, 1991; Hof *et al.*, 1995, 1996b). Corticocortical neuron populations that express high levels of SMI-32 include cells that project from polysensory association cortices (superior temporal sulcus) to prefrontal cortices and from V1, V2, V3 to MT (Campbell *et al.*, 1991; Hof *et al.*, 1995, 1996b). Equal numbers of corticocortical neurons that send their projections from high level visual association cortex (intraparietal sulcus) to prefrontal cortex express SMI-32 (Campbell *et al.*, 1991). Our results confirm previous findings in rats, which show that somatomotor corticocortical pyramidal neurons in layers III and V projecting contralaterally express very low levels of SMI-32 (Kirkcaldie *et al.*, 2002). One must, therefore, take into account that SMI-32 specificity may vary between primate and non-primate species.

Several studies have correlated the neurofilament amount or type with specific cellular populations in order to better understand the functional significance of each cell type. It is known that the amount of neurofilament increases with the increase of the cell size (Campbell and Morrison, 1989; Tsang *et al.*, 2000); therefore, layer V projection neurons would be expected to have high neurofilament levels compared to other cortical layers. Campbell and Morrison (1989) first showed that non-phosphorylated neurofilaments are found in neurons with long axonal projections extending to cortical and subcortical

areas. This finding is supported by studies that show high neurofilament levels in: long distance ipsilateral and contralateral cortex projections (Campbell *et al.*, 1991), long ipsilateral association connections of the visual system (Hof *et al.*, 1996a), large retinal ganglion cells (Straznicky *et al.*, 1992) and long corticofugal projections (Hornung and Riederer, 1999). Few cells having short corticocortical, callosal or limbic projections express neurofilament epitopes (Hof *et al.*, 1995, 1996a; Hornung and Riederer, 1999). Our observations demonstrate that layer V pyramidal neurons with callosal (type II) projections do not express these selected neurofilament epitopes, in spite of their long axons. However, it has been suggested that the neurofilament amount might not be associated with axonal length. Instead, the neurofilament quantity might be associated with the amount of axonal myelination (Kirkcaldie *et al.*, 2002). The degree of myelination corresponds with the projection target and would explain the contradictions between the different neurofilaments in ipsilateral connections and callosal connections. Cortical projections extending to subcortical targets and projections from primary sensory cortex extending to association areas (heterotopic) have heavily myelinated axons and are integral for important cognitive and motor processing. Goldstein *et al.* (1987) found that NF-H protects against proteolysis, thus increasing structural stability, which is critical in large, fast-conducting neurons. In addition to microtubules, neurofilaments have been implicated in regulating the nutrient transport rate, which is especially important in maintaining the structural integrity of these large neurons (Lasek, 1988).

We investigated whether these two distinct classes of layer V cells preserve their neurofilament specificity in two different organotypic slice culture paradigms, in which the projection neurons have been axotomized and lack contact with their targets. When E16 cortical slices were cultured for 2 weeks, SMI-32 and FNP-7 immunoreactive cells were still present, suggesting that the subpopulation phenotype is not dependent upon the target and is an inherent property of neurons. Obviously, before the connections reach their target, the back-labeling of selective layer V subpopulations is not possible; therefore, the issue of subpopulation specificity cannot be addressed with the techniques used in the current study at this embryonic stage. However, maintenance of subpopulation neurofilament specificity in the absence of target tissue contact can be addressed at later developmental stages. In this study, we demonstrated that after 2 weeks of culturing P4 slices, the overall pattern of SMI-32 and FNP-7 immunoreactivity was consistent with the *in vivo* observations. The pyramidal neurons labeled from the superior colliculus at P3 maintained specific expression of SMI-32 and FNP-7, but neurons labeled from the contralateral cortex hemisphere did not express these epitopes. These findings suggest that whilst the specific subpopulation phenotype development normally occurs once the axons have innervated their targets, this process is not dependent upon those targets. Therefore, it stands to reason that by P4, the two layer V neuronal subpopulations might already be committed to different differentiation programs which continue in the absence of their target tissue contact.

In summary, this study demonstrates that layer V neuronal subpopulations not only have specific projection targets and somatodendritic morphologies, but also express different structural protein levels recognized by SMI-32, NFP-7 and N200 antibodies. The finding that neurofilament proteins are expressed in subcortical (superior colliculus and spinal cord type I) projecting neurons (but not in type II, homotopic callosal projecting cells) that have large dendritic tufts supports theories that neurofilaments are associated with dendritic arborizations, long projecting axons and axons with a high degree of myelination. The characterization of molecular and cellular differences in adult rat layer V neuronal subpopulations sheds light on the mechanisms involved in functional circuit differentiation in mammalian cerebral cortex development. The specific neurofilament expression in certain neuronal populations, even in the absence of long projections and maintenance after distant target removal through slice culturing, suggests that early in corticogenesis, neuronal subpopulations are already committed to distinct protein expression patterns.

Notes

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Address correspondence to Dr Zoltán Molnár, Department of Human Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, OX1 3QX, UK. Email: zoltan.molnar@anat.ox.ac.uk.

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